

Des- γ -carboxyl prothrombin is associated with tumor angiogenesis in hepatocellular carcinoma

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Abstract

Background and Aims:

Hepatocellular carcinoma (HCC) is a hypervascular tumor, and angiogenesis plays an important role in its development. Previously, we demonstrated that des- γ -carboxyl prothrombin (DCP) promotes both cell proliferation and migration of human umbilical vein endothelial cells (HUVECs) by inducing the autophosphorylation of kinase insert domain receptor (KDR). In the present study, DCP-associated tumor angiogenesis was assessed by comparing hypovascular and common hypervascular HCC.

Methods:

The solitary HCCs of 827 patients were classified into 2 groups according to the tumor density at the arterial phase of a dynamic computed tomography scan; the initial clinical data of patients with the hyper- and hypovascular types were compared. The HCC tissues from 95 tumors were analyzed by immunohistochemical staining for DCP and phosphorylated KDR, and intratumoral microvessel density (MVD) was analyzed to evaluate microvessel angiogenesis.

Results:

The serum DCP levels (320 ± 3532 mAU/mL) and tumor size (18.4 ± 9.0 mm) of patients with hypervascular HCC were significantly greater than those with hypovascular HCC (38.7 ± 80 mAU/mL and 14.6 ± 5.2 mm, $P < 0.001$). Immunohistochemical analysis revealed that the expressions of DCP and phospho-KDR were significantly greater in hypervascular HCC (71.4% and 31.0%, respectively) than in hypovascular HCC (7.6% and 5.7%, respectively). Intratumoral MVD was significantly correlated with DCP ($r = 0.48$, $P < 0.0001$).

Conclusions:

DCP production is associated with tumor angiogenesis in HCC.

Keywords: Des- γ -carboxyl prothrombin; hepatocellular carcinoma; kinase insert domain receptor; intratumoral microvessel density

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common type of tumor worldwide and the third most common cause of cancer-related death ^{1,2}. For localized tumors, effective and palliative treatment options include surgical resection, liver transplantation, local ablation therapy, and transcatheter arterial chemoembolization. However, HCC is diagnosed at advanced stages in many patients ³⁻⁵. Although a few molecular-targeting agents have recently become clinically available, their effects are relatively limited ⁶. Therefore, new therapeutic targets for HCC are urgently needed to manage tumor progression.

HCC is a hypervascular tumor diagnosed according to a radiological finding of an arterial hypervascular pattern ^{5,7}. HCC progression is strongly related to active neovascularization ⁸⁻¹⁰, which promotes tumor growth by supplying oxygen and nutrients ¹¹. Angiogenesis is essential for tumor invasion and metastasis in addition to tumor growth ^{12,13}. Therefore, suppressing tumor angiogenesis can lead to the control of tumor progression. Several angiogenic factors including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), pituitary tumor-transforming gene 1, fibroblast growth factor (FGF), and angiopoietin-2 are associated with HCC tumor angiogenesis ¹⁴⁻²⁰.

Des- γ -carboxyl prothrombin (DCP) is a well-known tumor marker of HCC ^{7,21} whose expression is significantly correlated with poor prognosis ²²⁻²⁴. DCP is also a useful indicator of vascular invasion ^{22,25,26}. Previously, we demonstrated that DCP promotes both cell proliferation and migration in human umbilical vein endothelial cells (HUVECs) via the autophosphorylation of kinase insert domain receptor (KDR; also known as VEGF receptor 2) ²⁷. The tube formation of vascular endothelial cells is induced by DCP in a dose-dependent manner ²⁸. Furthermore, DCP treatment increases the expression of various angiogenic factors in HCC cells²⁹. Although

these studies demonstrate the potential paracrine function of DCP in HCC development, further investigation with clinical tissue samples is necessary.

Gadolinium-ethoxybenzyl-diethylenetriamine (Gd-EOB-DTPA) recently became a clinically available contrast agent for magnetic resonance imaging (MRI). Hypovascular HCC can be detected using hepatocyte-specific imaging with Gd-EOB-DTPA³⁰. Here, we evaluated the association between DCP production and HCC angiogenesis by comparing hyper- and hypovascular HCC. We compared the clinical data of patients with hyper- and hypovascular HCC and investigated the correlation between DCP expression and HCC angiogenesis in human HCC tissue.

Materials and Methods

Patients

A total of 827 patients who underwent radiofrequency ablation (RFA) or hepatic resection for solitary HCC (i.e., no metastasis) in Okayama University Hospital (Okayama, Japan) between January 2003 and October 2009 were enrolled. These patients had single space-occupying lesions without distant metastases according to imaging modalities such as ultrasonography (US), computed tomography (CT), angiography, and MRI. HCC was diagnosed according to the practice guidelines from the American Association for the Study of Liver Disease (AASLD)^{31, 32}. Atypical nodules and nodules <1 cm were histologically confirmed to be HCC by fine-needle aspiration biopsy under US guidance. In accordance with institutional guidelines, we obtained informed consent from all liver tissue sample donors and for their use of their clinical data. This study was approved by the Research Ethics Committee of Okayama University. All patients were Japanese and had chronic liver disease; 122 and 651 tested positive for hepatitis B surface antigen and hepatitis C virus antibodies, respectively. All blood tests were performed upon admission. The patients were classified into hyper- and hypovascular groups according to tumor vascularity evaluated in the arterial phase of a dynamic CT scan (Aquilion™; Toshiba, Tokyo, Japan). The arterial phases were acquired automatically starting 30 s after the intravenous bolus injection of contrast agents. If the hyperattenuation of the tumor at the arterial phase was observed by comparing the attenuation of the tumor to that of the hepatic parenchyma, the tumor was classified as hypervascular HCC. Other tumors were classified as hypovascular HCC. As mentioned above, all hypovascular HCCs underwent US-guided fine-needle biopsy for the diagnosis of hepatocellular carcinoma. Fourteen parameters obtained before the initial treatment were analyzed: age; sex; etiology; Child–Pugh class; the presence of ascites; the presence of

hepatic encephalopathy; tumor size; and the levels of serum biochemical and tumor markers—alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total bilirubin, prothrombin time activity, α -fetoprotein (AFP), and DCP.

HCC tissue and immunohistochemistry

Paraffin-embedded tissue sections were obtained from 95 HCC patients treated between 2003 and 2009 at Okayama University Hospital. In total, 53 HCC tissue specimens along with adjacent liver tissues were obtained by US-guided fine-needle liver biopsy and 42 by surgical resection. The 42 HCCs obtained by surgical resection were classified into the hypervascular group, and the 53 HCCs obtained by biopsy were classified into the hypovascular group. Immunohistochemistry was performed on dewaxed and dehydrated formalin-fixed paraffinized sections. After rehydration, endogenous peroxidase activity was blocked for 30 min using methanol solution containing 0.3% hydrogen peroxide. Nonspecific antibody binding was blocked by incubation with protein block serum-free solution (X0909; Dako Japan, Tokyo, Japan) for 30 min. The sections were incubated with primary antibodies against DCP (MU-3; Eisai Co. Ltd., Tokyo, Japan), phospho-KDR (2478; Cell Signaling Technology, Beverly, MA), and CD31 (M0823, Dako Japan) overnight at 4°C. Primary antibodies were detected using a biotinylated anti-rabbit secondary antibody (Dako Japan). The signal was amplified by avidin–biotin complex formation and developed with diaminobenzidine followed by hematoxylin counterstaining. The sections were subsequently dehydrated in alcohol and xylene, and mounted for observation. Sections were scored on a 4-titer scale: 0, negative; 1, weak signal; 2, intermediate signal; and 3, strong signal.

To determine microvessel density (MVD), the tissue sections were screened under low

magnification (40×) and the most vascularized areas within tumors were selected; the 3 selected areas were photographed with a digital camera under high magnification (100×) (BX51 and DP50; Olympus, Tokyo, Japan)³³. The recorded images had a resolution of 1,920,000 pixels. To calculate the MVD, the areas occupied by CD31-positive microvessels were quantified using Adobe Photoshop (version CS4; Adobe Systems Inc.). The average MVDs of the 3 selected areas were calculated as percentages of the CD31-stained area in a field of tumor sections. The sections were independently scored by two observers blinded to the groups the samples were from. We reviewed all discrepancies in the scoring process and reached a consensus on all sections.

Cell culture

The human HCC cell line Hep3B was obtained from the American Type Culture Collection (Manassas, VA). Hep3B cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO), 1% nonessential amino acids (Sigma), 1% sodium pyruvate (Sigma), and 1% penicillin/streptomycin solution (Sigma). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Quiescence was carried out under restricted serum conditions with 0.1% dialyzed FBS for the indicated time periods.

Stable transfection

To alter DCP production in Hep3B cells, γ -glutamyl carboxylase (GGCX) activity was modified by introducing wild-type (WT)-GGCX cDNA and exon 2 deletion variant (Δ 2)-GGCX cDNA, which has a dominant-negative effect³⁴. For stable transfection, Hep3B cells were

transfected with either pCEP4-WT-GGCX or pCEP4- Δ 2-GGCX using Superfect™ (Qiagen). Cells were selected by 400 μ g/mL G418 (Nakalai). Polyclonal lines consisting of more than 20 colonies were established. At least 2 independent stably transfected lines were established for each construct.

The DCP levels produced by the cell lines were determined by electrochemiluminescence immunoassay (ECLIA) (Picolumi PIVKA-II™; Sanko Junyaku Co. Ltd., Tokyo, Japan). The ECLIA uses a mouse monoclonal anti-DCP antibody coated on solid-phase beads and a ruthenylated rabbit polyclonal anti-prothrombin antibody. The electrochemically triggered light reaction was quantified by an electrochemiluminescence detection system (Roche Diagnostics, Basel, Switzerland). Cells were grown to confluence in 10-cm culture dishes and incubated with quiescent media for 24 h. The conditioned media were collected, and the DCP produced by each cell line was determined using ECLIA (Hep3B-WT-GGCX; undetectable, Hep3B- Δ 2-GGCX; 7.9 ng·mL⁻¹·day⁻¹·10⁻⁶ cells).

Tube formation assay

An in vitro tube formation assay was performed to investigate angiogenesis³⁵. The tube formation experiments were conducted in triplicate in 24-well dishes using an angiogenesis kit (Kurabo, Osaka, Japan) according to the manufacturer's instructions. Conditioned media from either Hep3B-WT-GGCX or Hep3B- Δ 2-GGCX cells were maintained in serum-free medium for 3 days without changing the medium. Conditioned media were collected and diluted (1:1) with the endothelial cell medium in the angiogenesis kit. The media were changed every 3 days. After 11 days, the dishes were washed with PBS and fixed with 70% ethanol at 4°C. The fixed cells were stained for CD31 using the Tubule Staining Kit (Kurabo). Luminal length was evaluated

using the Angiogenesis Image Analyzer (Kurabo) in 8 different fields for each culture.

Statistical analysis

Quantitative data are expressed as means \pm standard deviation. The Wilcoxon test, Fisher's exact probability test, the chi-squared test, and the Kruskal–Wallis test were used to evaluate the differences between the hyper- and hypovascular groups. ANOVA was used to assess the differences in serum DCP levels between the hyper- and hypovascular groups. Correlations were evaluated using the Spearman rank test. *P* values <0.05 on 2-tailed tests were considered significant.

Results

Patient profiles

The baseline characteristics of the patients are shown in Table 1. This study included 827 patients: 563 men and 264 women (median, 68 years; range, 34–90 years). All patients had single HCC, and the median tumor size was 15 mm. Liver function was assessed according to Child–Pugh class. Based on the arterial phase of dynamic CT, 675 and 152 patients had hyper- and hypovascular tumors, respectively. No parameters differed significantly between groups except a small but significant difference regarding the etiology of HCC ($P = 0.035$, chi-square test) due to the small number of patients with HBV/HCV co-infection.

Relationship between serum DCP level and HCC hypervascularity

Serum DCP levels (320 ± 3532 mAU/mL) and tumor size (18.4 ± 9.0 mm) in the hypervascular group were significantly greater than those (38.7 ± 80 mAU/mL and 14.6 ± 5.2 mm, respectively) in the hypovascular group (both $P < 0.0001$, Wilcoxon test). The tumor sizes were categorized as <10 , 11–20, 21–30, or >31 mm. Table 2 presents the relationship between serum DCP level and tumor size. Serum DCP levels were not significantly different in the <10 mm group, while those of the hypervascular group were significantly greater in the 11–20 ($P = 0.0052$) and 21–30 mm ($P = 0.039$) groups. In the >31 mm group, the serum DCP levels of the hypervascular group tended to be greater. In the hypervascular HCC group, serum DCP levels were significantly correlated with tumor size ($P < 0.001$, ANOVA); however, no significant correlation was observed in the hypovascular HCC group ($P = 0.75$).

Immunohistochemistry for DCP and phospho-KDR

The expressions of DCP and phospho-KDR were analyzed by immunohistochemistry in 42 and 53 HCCs in the hyper- and hypovascular groups, respectively. **Fig. 1** presents the DCP and phospho-KDR staining scores. The proportion of DCP expression in the hypervascular group (71.4%) was significantly greater than that in the hypovascular group (7.6%) ($P < 0.001$) (**Fig. 1C**). Furthermore, the proportion of phospho-KDR-positive tissue in the hypervascular group (31.0%) was significantly greater than that in the hypovascular group (5.7%) ($P = 0.02$) (**Fig. 1D**). There was a significant correlation between DCP and positive phospho-KDR expression ($r = 0.38$, $P = 0.0001$, Spearman's rank test).

Relationship between DCP expression and intratumoral MVD

Intratumoral MVD was assessed by measuring the CD31 staining of endothelial cells^{9,36}. The median intratumoral MVD was $1.6\% \pm 2.4\%$ (Table 3). The hypervascular group had a greater intratumoral MVD ($2.9\% \pm 3.0\%$) than the hypovascular group ($0.48\% \pm 0.44\%$) ($P < 0.0001$). Furthermore, DCP expression and intratumoral MVD were significantly correlated ($r = 0.48$, $P < 0.0001$, Spearman's rank test), and the intratumoral MVD was significantly greater in DCP-positive tumors ($2.9\% \pm 3.1\%$) than DCP-negative ones ($0.79\% \pm 1.3\%$, $P < 0.0001$). Moreover, moderately differentiated HCCs had greater intratumoral MVD (2.9 ± 3.0) than well-differentiated HCCs (0.78 ± 1.4). Well-differentiated HCCs had significantly greater intratumoral MVD in DCP-positive tumors ($2.2\% \pm 3.2\%$, $n = 10$) than DCP-negative ones ($0.51\% \pm 0.49\%$, $n = 51$, $P = 0.02$). Meanwhile, among moderately differentiated HCCs, DCP-positive tumors tended to exhibit greater intratumoral MVD ($3.3\% \pm 3.1\%$, $n = 24$) than DCP-negative ones ($2.2\% \pm 2.8\%$, $n = 10$, $P = 0.17$).

Tube formation assay

The biological effects of DCP were investigated by a tube formation assay using HUVECs co-cultured with fibroblasts. Conditioned medium from either Hep3B-WT-GGCX or Hep3B- Δ 2-GGCX was added to HUVECs and fibroblasts plates. The conditioned medium from Hep3B- Δ 2-GGCX activated the tube formation of HUVECs (**Fig. 2A**). The luminal length of HUVEC tubules with the conditioned medium from Hep3B- Δ 2-GGCX was significantly greater than that with the conditioned medium from Hep3B-WT-GGCX (**Fig. 2B**).

Discussion

By comparing hyper- and hypovascular tumors, we demonstrated that DCP production is associated with tumor angiogenesis. We and others previously demonstrated that DCP stimulates vascular endothelial cell proliferation and migration ^{27,28}. However, the effects of DCP production in clinical tissues remained unknown until now. The present results clearly demonstrate the relationship between DCP production and angiogenesis in HCC tissue for the first time.

Gd-EOB-DTPA, a recently developed contrast agent for MRI imaging, enables the detection of small nodules diagnosed as hypovascular HCC, which were previously difficult to detect ³⁰. Golfieri et al. demonstrate that the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI is useful for detecting hypovascular HCC ³⁷. Although nodules with atypical vascular patterns need to be diagnosed by biopsy, Gd-EOB-DTPA-enhanced MRI improves early hypovascular HCC detection. In solitary HCC (no metastasis), the serum DCP levels and tumor sizes were significantly greater in the hypervascular group (Table 1). Previously, we demonstrated that DCP acts as an autologous growth factor for HCC and stimulates the Met-JAK-STAT signaling pathway ³⁸. Because serum DCP levels may increase with respect to tumor volume, we classified HCC according to tumor size. In the hypervascular HCC group, serum DCP levels increased significantly with tumor size (Table 2), whereas this was not the case in the hypovascular HCC group (Table 2). After adjusting for tumor size, the serum DCP levels of the hypervascular HCC group were greater than those of the hypovascular HCC group. In the >31 mm group, statistical significance was unclear because of the small number of hypovascular HCCs >31 mm; large hypovascular HCCs are rare because angiogenesis is essential for tumor growth ³⁹.

The immunohistochemical analysis revealed that the expressions of DCP and phospho-KDR

were greater in the hypervascular group than the hypovascular group (**Fig. 1**). This suggests that DCP plays an important role in HCC angiogenesis. In addition, KDR phosphorylation is important for the angiogenesis of HCC. Previously, we demonstrated that DCP directly induces KDR autophosphorylation and activates the KDR-PLC- γ -MAPK signaling pathway without VEGF involvement ²⁷. Thus, the present results demonstrate a positive correlation between DCP and phospho-KDR expression in clinical tissue samples.

Vasculogenic mimicry (i.e., tumor cells that mimic the patterns of vasculogenic networks) was recently reported to be implicated in HCC tumor angiogenesis ⁴⁰. The immunohistochemical analysis for CD31 expression revealed that intratumoral MVD in the hypervascular group was greater than that in the hypovascular group (Table 3). We and others demonstrated that DCP promotes vascular endothelial cell proliferation and migration in vitro ^{27,28}. Accordingly, DCP expression was correlated with intratumoral MVD in the clinical specimens.

Although the proportion of phospho-KDR expression in the hypervascular group was greater than that in the hypovascular group, it was relatively low compared to the proportion of DCP-positive expression. This may be due to the sensitivity of immunostaining for phospho-KDR. In addition, tumor angiogenesis could also be regulated by other angiogenic factors including VEGF, PDGF, FGF, and angiopoietin-2 ¹⁴⁻²⁰.

One limitation of the present study is that tumor vascularity was evaluated by dynamic CT scans instead of histology. Tumor vascularity may be underestimated when new blood vessels are still low in the dynamic CT scan, even if tumor angiogenesis occurred at the histological level. Therefore, tumor vascularity was evaluated by histology (Table 3). The results regarding intratumoral MVD are concordant with those of the dynamic CT scans. Thus, we carefully adjusted the staining density of the fine-needle biopsy specimens and surgically resected tissue

samples by using biopsy specimens from the hypervascular HCCs.

Furthermore, there may be a potential bias due to the sampling method of the specimens; most tissue specimens from the hypervascular group were obtained by surgical resection while those from the hypovascular group were obtained by fine-needle biopsy. However, this limitation is inevitable if we follow the AASLD's HCC practice guidelines^{31, 32}. Consequently, further matched-pair case-control studies may be necessary.

Many angiogenic and angiostatic factors are associated with tumor angiogenesis. Therefore, it was necessary to clarify whether DCP induces tumor angiogenesis without the help of other angiogenic factors. The enzyme GGCX converts DCP into normal prothrombin with vitamin K epoxide reductase⁴¹. Previously, we demonstrated that DCP-positive HCC cell lines express $\Delta 2$ -GGCX, which attenuates GGCX activity via a dominant-negative effect³⁴. The comparative study of HCC cell lines with WT-GGCX and $\Delta 2$ -GGCX allows us to evaluate the angiogenic effect of DCP production while excluding the effects of other angiogenic factors. In the tube formation assay, the angiogenic activity of the conditioned medium from Hep3B- $\Delta 2$ -GGCX was significantly greater than that from Hep3B-WT-GGCX. This result is consistent with the tube formation induced by purified DCP using HUVECs²⁸. The conditioned medium from Hep3B-WT-GGCX induced tube formation, suggesting DCP is not the only angiogenic factor at work. As mentioned above, angiogenesis is regulated by other angiogenic factors such as VEGF, PDGF, FGF, and angiopoietin-2¹⁴⁻²⁰.

In conclusion, we clinically demonstrated that DCP production is associated with tumor angiogenesis as evidenced by differences in vascularity. HCC tumor angiogenesis is associated with risks of invasion¹², metastasis^{10, 12}, poorer disease-free survival, and early recurrence after HCC resection⁴². Therefore, DCP may be a useful therapeutic target for controlling the tumor

angiogenesis of HCC and requires further study.

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Tables

Table 1. Baseline characteristics

	Hypervascular group (n = 675)	Hypovascular group (n = 152)	<i>P</i> value
Age	71.0 ± 9.5	71.9 ± 8.3	0.53 [†]
Sex (M/F)	470/205	93/59	0.053 [‡]
Etiology (HBV/HCV/HBV+HCV/other)	(97/509/10/59)	(14/131/1/6)	0.035 ^{§*}
Child–Pugh class (A/B/C)	(528/120/6)	(128/23/0)	0.67 [¶]
Total bilirubin level (g/dL)	0.97 ± 0.50	0.97 ± 0.41	0.37 [†]
Albumin level (mg/dL)	3.67 ± 0.54	3.68 ± 0.47	0.90 [†]
Prothrombin time activity (%)	92.3 ± 17.9	92.8 ± 17.2	0.65 [†]
Ascites (none/mild/severe)	(533/96/7)	(133/16/2)	0.25 [¶]
Hepatic encephalopathy (0/grade I and II/grade III and IV)	(644/12/0)	(148/3/0)	0.90 [¶]
AST level (IU/L)	54.9 ± 28.6	58.2 ± 35.3	0.19 [†]
ALT level (IU/L)	47.7 ± 28.9	50.0 ± 32.2	0.51 [†]
AFP level (ng/mL)	196 ± 2165	45.8 ± 119	0.17 [†]
DCP level (mAU/mL)	320 ± 3532	38.7 ± 80	<0.0001 ^{†*}
Tumor size (mm)	18.4 ± 9.0	14.6 ± 5.2	<0.0001 ^{†*}

Data are means ± SD. *P* values were calculated using [†]the Wilcoxon test, [‡]Fisher's exact

probability test, [§]the chi-square test, and [¶]the Kruskal–Wallis test. **P* < 0.05 was considered

significant.

Table 2. Relationship between primary tumor size and serum DCP levels

Tumor size (mm)	DCP level (mAU/mL)		<i>P</i> value
	Hypervascular group (n = 675)	Hypovascular group (n = 152)	
0–10 (n = 133)	41.1 ± 131 (n = 100)	29.0 ± 32.3 (n = 33)	0.44
11–20 (n = 486)	72.1 ± 223 (n = 383)	43.4 ± 94.2 (n = 103)	0.0052*
21–30 (n = 145)	135.7 ± 260 (n = 132)	21.9 ± 12.3 (n = 13)	0.039*
31+ (n = 63)	2771 ± 11610 (n = 60)	39.0 ± 15.5 (n = 3)	0.17

Data are means ± SD. *P* values were calculated using the Wilcoxon test.

Table 3. Relationship between DCP expression and vascularization

Group	Number of HCC samples	MVD (%) in tumor		<i>P</i> value
Vascular type				
Hypervascular	42	2.9 ± 3.0	}	<0.0001
Hypovascular	53	0.48 ± 0.44		
DCP expression				
Positive	34	2.9 ± 3.1	}	<0.0001
Negative	61	0.79 ± 1.3		
Tumor differentiation				
Well differentiated	61	0.78 ± 1.4	}	<0.0001
Moderately differentiated	34	2.9 ± 3.0		

Data are means \pm SD. *P* values were calculated using the Wilcoxon test.

Figure Legends

Fig. 1. Immunohistochemistry of DCP and phospho-KDR

(A, B) Immunohistochemical staining for DCP (A) and phospho-KDR with protein scores of 3

(B) In HCC (original magnification, 100×).

(C, D) The sections of each bar represent the percentage of each group with a particular score: 0–3 for DCP (C) and phospho-KDR (D).

Fig. 2. Tube formation assay with the conditioned media of HCC cell lines

Equal amounts of conditioned media from the HCC cultures were added to the basal culture medium in the HUVEC/fibroblast co-culture system. Cells were stained with anti-CD31 antibody.

(A) Representative photographs of tube formation. (B) Luminal length was quantified with the Angiogenesis Image Analyzer (Kurabo, Osaka, Japan). Data are expressed as means \pm SD. control–WT-GGCX, $*P = 0.0005$ and WT-GGCX– $\Delta 2$ -GGCX, $**P = 0.004$.